PB86-107232

Identification of Mutagenic Compounds Formed during Chlorination of Humic Acid

(U.S.) Health Effects Research Lab.
Cincinnati, OH

1985

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA (Please read Instructions on the reverse before completir		
1. REPORT NO. EPA/600/J-85/164	2.	3. F PB86-107232
4. TITLE AND SUBTITLE Identification of Mutagenic Compounds Formed During Chlorination of Humic Acids (Journal Version)		5. REPORT DATE 1985 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) John R. Meier, H. Paul Ringhand, W. Emile Coleman, Jean W. Munch, Robert P. Streicher, William H. Kaylor*		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Toxicology and Microbiology Division Health Effect Research Laboratory, ORD U.S. Environmental Protection Agency Cincinnati, OH 45268		10. PROGRAM ELEMENT NO. ABMC1A 11. CONTRACT/GRANT NO. Inhouse
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, North Carolina 27711		13. TYPE OF REPORT AND PERIOD COVERED Journal 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES P.O. John R. Meier Mutation Research 157, 111-122, August/September 1985 *and Kathy Schenck		

16. ABSTRACT

Humic acid chlorination products are being studied in an effort to identify the chemicals responsible for the mutagenicity formed during water chlorination. In the present report, nineteen chlorinated organic compounds have been identified and quantified in ether extracts of chlorinated humic acid solutions. Ten of these compounds, including a number of chlorinated propanones and chlorinated propenals, are direct-acting mutagens in the Salmonella/microsome mutagenicity assay. The position of the chlorine substituent has been found to be an important factor in the mutagenic activity of these two classes of compounds. The total mutagenicity of the compounds identified thus far, when tested either individually or as a composite, accounts for only about 8% of the total mutagenicity formed during humic acid chlorination. The addition of bromide to the humic acid chlorination reaction results in up to a two-fold increase in the level of mutagenicity formed.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS (This Report) unclassified	21. NO. OF PAGES 14
	20. SECURITY CLASS (This page) unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

This article documents research that was conducted under the auspices of the U.S. Environmental Protection Agency (USEPA). As an arm of the government, the USEPA has a paid up, non-exclusive, irrevocable, worldwide license to reproduce, prepare derivative works, and distribute to the public all of the material contained herein, and to grant others acting on its behalf the right to do so. The USEPA grants to the National Technical Information Service, Department of Commerce, the right to act as its agent in the distribution of this material.

EPA/600/J-85/164
JOURNAL ARTICLE

Mutation Research, 157 (1985) 111–122
Elsevier

MTR00990

Identification of mutagenic compounds formed during chlorination of humic acid

John R. Meier, H. Paul Ringhand, W. Emile Coleman, Jean W. Munch, Robert P. Streicher, William H. Kaylor and Kathy M. Schenck

U.S. Environmental Protection Agency, Health Effects Research Laboratory, Toxicology and Microbiology Division, 26 West St. Clair, Cincinnatti, OH 45268 (U.S.A.)

(Received 26 October 1984)
(Revision received 2 February 1985)
(Accepted 21 February 1985)

Summary

Humic acid chlorination products are being studied in an effort to identify the chemicals responsible for the mutagenicity formed during water chlorination. In the present report, 19 chlorinated organic compounds have been identified and quantified in ether extracts of chlorinated humic acid solutions. 10 of these compounds, including a number of chlorinated propanones and chlorinated propenals, are direct-acting mutagens in the Salmonella/microsome mutagenicity assay. The position of the chlorine substituent has been found to be an important factor in the mutagenic activity of these two classes of compounds. The total mutagenicity of the compounds identified thus far, when tested either individually or as a composite, accounts for only 7–8% of the total TA100 mutagenicity,and less than 2% of the TA98 mutagenicity formed during humic acid chlorination. The addition of bromide to the humic acid chlorination reaction results in up to a 2-fold increase in the level of mutagenicity formed.

The presence of mutagenic activity in drinking water has been well documented for samples taken from a variety of locations, and the subject has been reviewed in detail (Loper, 1980; Nestmann, 1983). Although a number of compounds with known mutagenic and carcinogenic properties have been identified in drinking water (Simmon et al., 1977; Bull, 1982; Kool et al., 1983; Tabor, 1983), little success has been attained in attributing the mutagenicity levels in water samples to specific chemicals present. Consequently, it is not yet known whether any or all of the chemicals identified thus far contribute significantly to the overall mutagenic activity of drinking water.

There is substantial evidence to indicate that water chlorination is responsible for the majority of the mutagenic activity present (Cheh et al., 1980; Maruoka and Yamanaka, 1980; deGreef et al., 1980; Fallon and Fliermans, 1980; Loper et al., 1985). Natural aquatic humic materials are likely precursors to the mutagens produced during water chlorination since they account for the bulk of the organic materials in source waters and are known to be precursors to trihalomethanes and other halogenated organic compounds which are present following chlorination (Rook, 1976; Stevens et al., 1978; Christman et al., 1980; Coleman et al., 1984). Recently, it has been shown that direct-acting mutagenic activity, detectable in the Salmonella/microsome assay, is formed following chlorination of humic materials (Bull et al., 1982; Meier et al.,

Corresponding author: John R. Meier, U.S. Environmental Protection Agency, Health Effects Research Laboratory, Toxicology and Microbiology Division, 26 West St. Clair, Cincinnati, OH 45268, U.S.A.

1983). The properties of the mutagenicity formed (i.e., non-volatile, alkaline labile, direct-acting activity, decreased by addition of S9, highest responsiveness in strains TA98 and TA100) are similar to those commonly observed for mutagenic activity isolated from drinking water samples. On the basis of these earlier findings, we have proposed the use of humic acid as a model substrate for studying the mutagenic components of drinking water (Meier et al., 1983). This rationale has received support from studies showing the similarity in compounds identified in chlorinated humic acid solutions with those identified in drinking water samples (Coleman et al., 1984; Kopfler et al., 1984). Recently, Kringstad et al. (1983) have reported the formation of a number of chloropropanones as well as 2-chloropropenal following humic acid chlorination, and have suggested that the latter compound might be an important contributor to the mutagenicity present. The results reported here do not support this conclusion.

In the present investigation, a number of mutagenic compounds formed during humic acid chlorination are identified and the contributions of the identified mutagens to the total mutagenicity present are discussed. Determinations of mutagenic activity in the Salmonella microsome assay have been performed for several previously unreported compounds including 1,1-dichloropropanone, 1,1,1-trichloropropanone, and 3,3-dichloropropenal.

Materials and methods

Chemicals

Commercially available chemicals were obtained from the following sources: humic acid, and 1,3-dichloropropanone from Fluka AG (Buchs, Switzerland); chloroacetonitrile, trichloroacetonitrile, 1,1-dichloropropanone, 1,1,3,3,-tetrachloropropanone, pentachloropropanone, dichloroacetic acid, and hexachlorocyclopentadiene from Aldrich Chemical Co. (Milwaukee, WI); dichloroacetonitrile from Pfaltz and Bauer (Stamford, CT); 2,4,6-trichlorophenol from Eastman Kodak Co. (Rochester, NY); hexachloroethane from Chem Service, Inc. (West Chester, PA); and trichloroacetic acid from Fisher Scientific (Fair lawn, NJ).

1,1,1-Trichloropropanone was sythesized by chlorination of 1,1-dichloropropanone (Wyandotte Chemicals Corp., 1965). 3,3-Dichloropropenal was synthesized by methods described elsewhere (Coleman et al., 1984). 2,3,3-Trichloropropenal was prepared as follows. Cl_2 was slowly added to 3,3-dichloropropenal in an ice bath until a substantial excess of Cl_2 had been added. The product formed (2,3,3,3-tetrachloropropanal) was purged with N_2 until the green color disappeared, and then added to distilled water at a ratio of 1:10. The mixture was heated to near boiling for 1 h and then steam distilled. The yellow lower layer of the distillate was separated and distilled under vacuum to yield 2,3,3-trichloropropenal. 2-Chloropropenal was synthesized as follows. In an ice bath, Cl_2 was bubbled slowly into acrolein. The resulting 2,3-dichloropropenal was added to 10 parts water and steam distilled. The lower layer of the distillate was washed with water, dried over magnesium sulfate, and distilled to give 2-chloropropenal.

Preparation and extraction of chlorinated humic acid samples

Humic acid solutions were prepared at a concentration of 1 g total organic carbon (TOC) per liter of distilled water, and chlorination was performed at pH 7, using a 1:1 ratio of chlorine equivalents per mole of carbon. The detailed procedures have been described previously (Meier et al., 1983). For experiments examining the influence of bromide on mutagen formation, potassium bromide was added during chlorination in amounts corresponding to Br to Cl molar ratios of 0, 0.01, 0.05, 0.1, 0.5, and 1.0.

A liquid–liquid extraction method with diethyl ether was used to recover mutagenic compounds in a solvent suitable for subsequent chemical identification. An internal standard, 25 μg of *p*-bromofluorobenzene, was added only to samples being extracted for chemical analysis. 200 ml of aqueous chlorinated humic acid and 150 ml of freshly distilled diethyl ether were shaken in a separatory funnel for 2 min, and the phases were separated by centrifugation. The ether layer was passed through a column (12 cm × 2.5 cm i.d.) of anhydrous sodium sulfate to remove residual water. The remaining aqueous layer was extracted a second time with 100 ml of ether, the extract passed through sodium sulfate, and combined with the

first extract. The combined ether extracts were concentrated to 1 ml by Kuderna Danish evaporation and analyzed by GC/MS. Residual ether remaining in the aqueous phase was evaporated with a stream of N_2 prior to analysis of this fraction for mutagenicity. A portion of the ether extract was diluted 200-fold in 10% acetone in H_2O, prior to mutagenicity analysis of this fraction.

Dichloroacetic acid and trichloroacetic acid were analyzed as their methyl esters using a modified extraction procedure. 25 μg of *p*-bromofluorobenzene were added to a 20-ml volume of the aqueous chlorinated humic acid. The sample was extracted with 2 portions, 15 and 10 ml respectively, of distilled diethyl ether. Concentration steps were unnecessary because of the high levels of the compounds present and were eliminated. A 1-ml portion of the resulting combined extract was methylated with gaseous diazomethane, and analyzed by GC/MS.

Assay for mutagenic activity

The methods used for mutagenicity assays were based on those described by Ames et al. (1975). *Salmonella tyhpimurium* strains TA1535, TA1537, TA98, and TA100 were a gift of Dr. Bruce Ames (Berkeley, CA). Strain-specific markers (i.e., ampicillin, crystal violet, and UV sensitivity; histidine dependence; spontaneous mutation rate; response to positive control chemicals) were confirmed for each strain prior to use. Assays were performed with duplicate or triplicate plates per dose level tested. Solvent, negative and positive controls were included in each assay. In assays employing S9 activation, the S9 fraction was added at a level of 50 μl of S9 per ml of S9 mix. The S9 fraction was prepared from livers of Aroclor-1254-pretreated, male, Sprague–Dawley rats according to the procedure of Ames et al. (1975). The general procedure was to conduct initial range-finding assays over a 3–4 log dose range in 1/2 log increments, and then to repeat the assay for positive chemicals over a narrower range to obtain a linear dose response. Mutagenicity levels were estimated from the linear portions of the dose–response curves (initial slopes) by least-square regression analysis of the data.

Capillary GC/MS system conditions and analyses

GC/MS analyses were performed on a Finnigan 3300 quadrupole mass spectrometer equipped with an Incos 2300 data system. A Carlo-Erbo fractovap 4160 gas chromatograph was equipped with a splitless injector and a 30 m × 0.25 mm i.e. fused silica DB1701 capillary column. A 2-μl sample was injected at 20°C in the splitless mode, and the split opened after 30 sec. The temperature was held at 20°C for 6 min, then the temperature was programmed at a rate of 6°/min to 250°C. The MS scan range was 14–450 amu and the rate was 1 scan/sec.

GC/MS quantitation

All compounds for which authentic standards were available were spiked into, and extracted from, a chlorinated humic acid sample in order to assess their recovery from the matrix. In order to decide upon a spiking amount for each compound, the concentration of each compound in the sample was estimated by comparison of its GC peak area with the area of the internal standard (IS), *p*-bromofluorobenzene. After spiking amounts were determined, stock solutions were prepared in acetone. Appropriate amounts of the stock solutions were added to a 200-ml chlorinated humic acid sample and extracted as previously described. All the standard compounds were also analyzed as dilutions of the acetone stocks in diethyl ether to determine their GC/MS response. A response factor (RF) for each compound (Z) was calculated using the equation:

$$RF = \frac{\text{single ion area of Z} \times \text{IS amount}}{\text{single ion area of IS} \times \text{amount Z}}$$

After samples, spikes, and standards had been analyzed, recovery efficiency was determined using the equation:

$$\text{Recovery efficiency} = (Y - X)/T$$

where T is the spike level, Y is the measured concentration of a spiked aliquot, and X is the background concentration (i.e., the amount in the sample before spiking). Recovery efficiencies were greater than 0.85 for all compounds except trichloroacetic acid, dichloroacetic acid, trichloroacetonitrile, and hexachloroethane, which were

0.77, 0.47, 0.40, and 0.31 respectively. The amount of each compound in the ether extract was calculated with the equation:

$$\text{Amount } Z_{(\text{extract})} = \frac{\text{single ion area } Z \times \text{IS amount}}{\text{single ion area of IS} \times RF}$$

The amount of each compound in the original chlorinated humic acid sample was calculated with the equation:

$$\text{Amount } Z_{(\text{Cl humic})} = \frac{\text{amount } Z_{(\text{extract})}}{\text{recovery efficiency} \times \text{concentration factor}}$$

where concentration factor is the level of concentration of the extracts (i.e., 1 for dichloroacetic acid and trichloroacetic acid, and 200 for the remaining compounds).

Results

Extraction and identification of mutagenic components

In previous work diethyl ether was found to be an effective solvent for extraction of mutagenic activity from chlorinated humic acid solutions (Coleman et al., 1984). In the present study, the extraction procedure was modified slightly, and the degree of extract concentration by Kuderna–Danish evaporation was increased from 5-fold to 200-fold in order to increase the detection sensitivity for identification of compounds present. An additional modification involved the addition of acetone (10% v/v in distilled H_2O) to the extracts rather than dimethyl sulfoxide (DMSO) upon dilution from 200 × to 1 × prior to mutagenicity testing. The acetone was found to improve the solubility of the extracted material upon readdition to water. DMSO was avoided as a transfer solvent because of the possibility of production of mutagenic reaction products which have been reported to occur as a result of reaction of DMSO with trichloroactic acid (Nestmann et al., 1980), and chloropropanones (Douglas et al., 1984), compounds known to be present in chlorinated humic acids (Coleman et al., 1984). Using these modified extraction conditions, the ability of diethyl ether to extract the mutagenicity present can be seen from the results in Fig. 1. It is clear that essentially all of the mutagenic activity, both for strains TA98 and TA100, was extractable with ether. No mutagenic activity was detectable in either the aqueous or ether layers upon extraction of a distilled water blank (data not shown). Total organic halogen (TOX) measurements were also conducted on the chlorinated humic acid samples, before and after ether extraction. The TOX levels were determined to be : 358 mg/l in the original sample, 278 mg/l in the aqueous phase after extraction, and 90 mg/l in the ether phase after extraction. Thus, in contrast to the efficient extraction of mutagenic activity, only about 25% of the total chlorinated organics were extractable with ether.

GC/MS analysis of the same ether extract used for mutagenicity evaluation resulted in the identification and quantification of a number of halogenated organic compounds, several of which have been reported to be mutagenic in the Ames

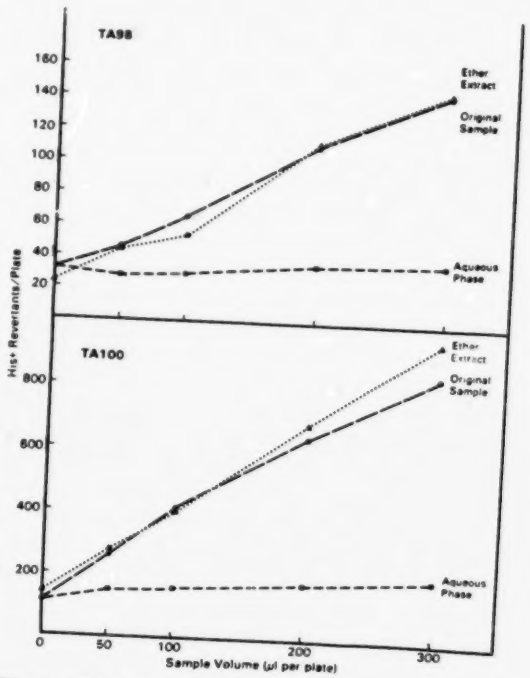

Fig. 1. Diethyl ether extraction of mutagenic activity in chlorinated humic acid solutions.

test (Table 1). The three most abundant compounds among those identified, chloroform, dichloroacetic acid, and trichloroacetic acid, have been previously reported to be non-mutagenic. However, the fourth most abundant compound, 1,1,1-trichloropropanone, had not been tested for mutagenic activity, according to information provided by the Environmental Mutagen Information Center (B. Whitfield, personal communication). Therefore, this compound was synthesized and tested for mutagenic activity in the Ames test. The results (Fig. 2) revealed clear dose-related increases in mutagenic activity for strains TA1535, TA98, and TA100. S9 activation was not required for detection of activity but some S9 enhancement of TA98 mutagenesis was observed. Two other identified compounds whose mutagenicity was unknown, 1,1-dichloropropanone and 3,3-dichloropropenal, were also assayed in the Ames test. The results (Fig. 3) showed that 1,1-dichloropropanone was active for strains TA1535 and TA100, but not for strain TA1537 or TA98. S9 was not required for the activity but the addition of S9 did appear to decrease the toxicity which was apparent at higher doses. The dose–response curves for 3,3-dichloropropenal mutagenicity are shown in Fig. 4. This compound was directly active for strains TA1535, TA100, and TA98. In this case the addition of S9 substantially decreased the mutagenic activity present.

In order to determine to what extent the identified mutagens might account for the total mutagenicity present in the chlorinated humic acid solutions, dose-response mutagenicity assays were conducted on the positive compounds in Table 1 to generate quantitative data on the mutagenicity of these compounds. Assays were performed without S9 with strains TA98 and TA100 since the

TABLE 1

QUANTIFICATION OF COMPOUNDS IN ETHER EXTRACTS OF CHLORINATED HUMIC ACID SOLUTIONS

Compound	Conc. [a] in extract (μg/ml)	Conc. in original solution (μg/ml)	Reported mutagenicity Ames test	References
Chloroform [b]	6000	30	–	Simmon et al., 1977
Trichloroacetonitrile [b]	91	1.1	–	Unpublished data
2-Chloropropenal	< 10	< 0.05	+	Rosen et al., 1980
1-Chloropropanone [b,c]	30	0.15	–	Cheh and Carlson, 1981
1,1-Dichloropropanone [b]	200	1.0	+	Present study
Chloroacetonitrile	2.8	0.01	–	Unpublished data
Dichloroacetonitrile [b]	900	5.3	+	Simmon et al., 1977
1,1,1-Trichloropropanone [b]	1960	9.8	+	Present study
1,3-Dichloropropanone	30	0.15	+	Kringstad et al., 1981
1,1,3-Trichloropropanone [d]	9	0.05	+	Douglas et al., 1983
1,1,1,3-Tetrachloropropanone [d]	10	0.05	?	–
1,1,3,3-Tetrachloropropanone [b]	530	2.6	+	Douglas et al., 1983
Pentachloropropanone	1260	7.2	+	Douglas et al., 1983
Hexachlorocyclopentadiene [b]	15	0.08	–	Haworth et al., 1983
2,4,6-Trichlorophenol [b]	14	0.07	–	Haworth et al., 1983
Hexachloroethane [b]	2.5	0.04	–	Haworth et al., 1983
Dichloroacetic acid [b]	1530	17	–	Waskell, 1978
Trichloroacetic acid [b]	4000	54	–	Waskell, 1978
3,3-Dichloropropenal	12	0.06	+	Present study
2,3,3-Trichloropropenal	7	0.035	+	Rosen et al., 1980

[a] Values based on triplicate determinations
[b] Compounds that have been identified in finished drinking water.
[c] Quantification based on 1,1-dichloropropanone.
[d] Quantification based on 1,1,3,3-tetrachloropropanone.

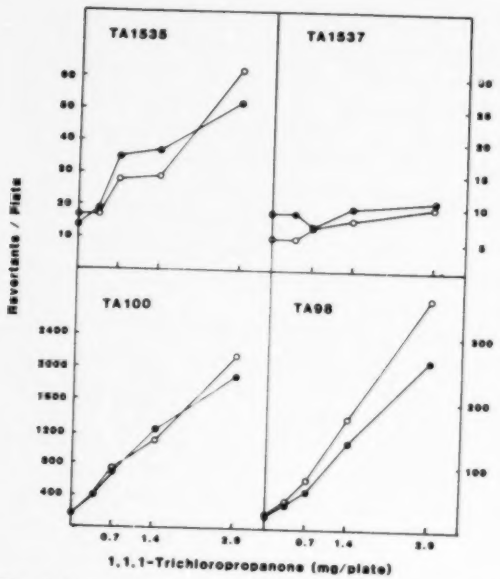

Fig. 2. Mutagenic activity of 1,1,1-trichloropropanone in the Salmonella/microsome assay. ○, with S9; ●, without S9.

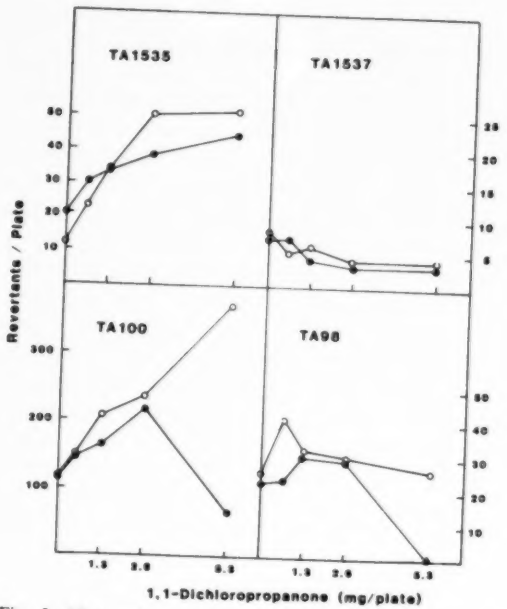

Fig. 3. Mutagenic activity of 1,1-dichloropropanone in the Salmonella/microsome assay. ○, with S9; ●, without S9.

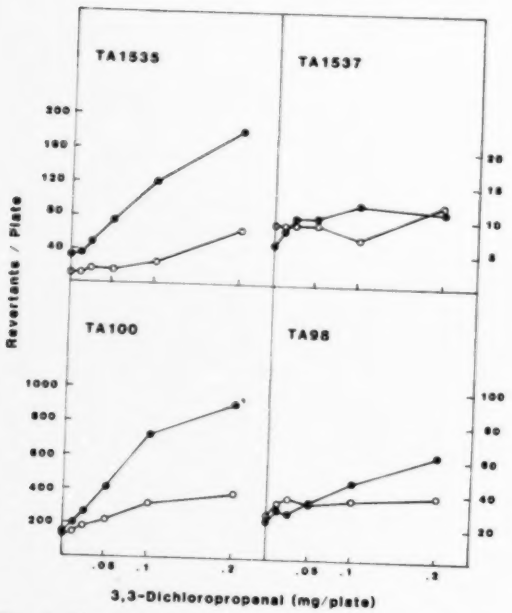

Fig. 4. Mutagenic activity of 3,3-dichloropropenal in the Salmonella/microsome assay. ○, with S9; ●, without S9.

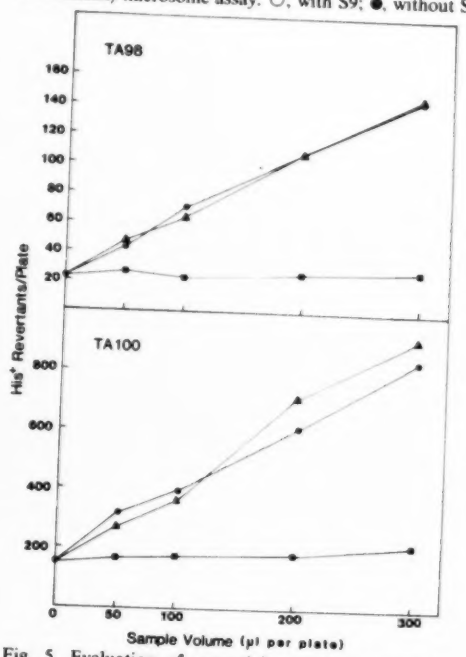

Fig. 5. Evaluation of potential synergism among identified components of chlorinated humic acid solutions. ■, identified compounds in H_2O; ●, chlorinated humic acid; ▲, chlorinated humic acid with added compounds.

TABLE 2

DIRECT-ACTING MUTAGENICITY OF IDENTIFIED CONSTITUENTS IN CHLORINATED HUMIC ACID SOLUTIONS FOR STRAINS TA98 AND TA100

Compound	Purity	Dose level (μg/plate)	His$^+$ revertants/plate [a] ± S.D.		Net revertants/μg [b]	
			TA98	TA100	TA98	TA100
Dichloroacetonitrile (CAS No. 3018-12-0)	> 99% [c]	0	15 ± 0	92 ± 5	2.30×10^{-2} ($\pm3.00\times10^{-3}$)	6.45×10^{-1} ($\pm3.80\times10^{-2}$)
		85	18 ± 3	164 ± 11		
		171	20 ± 10	205 ± 16		
		342	24 ± 9	324 ± 26		
		685	33 ± 5	425 ± 1		
		1369	48 ± 6	598 ± 26		
1,1-Dichloropropanone (CAS No. 513-88-2)	98% [c]	0	21 ± 5	113 ± 6	NS	3.80×10^{-2} ($\pm3.47\times10^{-3}$)
		664	23 ± 1	145 ± 13		
		1327	30 ± 8	165 ± 12		
		2654	29 ± 9	217 ± 18		
		5308	0 ± 0	66 ± 59		
1,3-Dichloropropanone (CAS No. 534-07-6)	> 98% [d]	0	17 ± 2	92 ± 10	NS	1.14×10^{2} ($\pm0.06\times10^{2}$)
		1.25	18 ± 4	257 ± 23		
		2.50	23 ± 0	358 ± 22		
		5.00	28 ± 6	671 ± 45		
		10.00	24 ± 4	862 ± 47		
1,1,1-Trichloropropanone (CAS No. 918-00-3)	> 98% [c]	0	22 ± 4	139 ± 16	8.75×10^{-2} ($\pm4.90\times10^{-3}$)	7.53×10^{-1} ($\pm1.13\times10^{-2}$)
		359	38 ± 2	389 ± 22		
		718	61 ± 3	690 ± 19		
		1435	137 ± 22	1211 ± 21		
		2870	264 ± 38	1874 ± 47		
1,1,3,3-Tetrachloropropanone (CAS No. 632-21-3)	97% [e]	0	14 ± 3	136 ± 9	5.65×10^{-1} ($\pm2.93\times10^{-2}$)	7.79 ($\pm9.69\times10^{-1}$)
		20	24 ± 3	313 ± 27		
		40	38 ± 4	448 ± 82		
		80	59 ± 4	569 ± 89		
		160	87 ± 13	–		
		320	95 ± 34	–		
Pentachloropropanone (CAS No. 1768-31-6)	98% [e]	0	32 ± 11	84 ± 10	4.28×10^{-1} ($\pm1.90\times10^{-2}$)	3.71 ($\pm6.59\times10^{-1}$)
		21	32 ± 10	179 ± 27		
		42	53 ± 5	240 ± 54		
		84	69 ± 10	294 ± 97		
		168	103 ± 14	387 ± 42		
		336	174 ± 4	397 ± 102		
3,3-Dichloropropenal (CAS No. 2648-51-3)	> 98% [f]	0	23 ± 5	141 ± 6	1.94×10^{-1} ($\pm1.98\times10^{-2}$)	5.83 ($\pm2.01\times10^{-1}$)
		12.5	29 ± 3	196 ± 7		
		25.0	27 ± 2	265 ± 10		
		50.0	34 ± 5	408 ± 18		
		100.0	46 ± 12	718 ± 61		
		200.0	62 ± 7	902 ± 110		
2,3,3-Trichloropropenal (CAS No. 3787-28-8)	> 98% [f]	0	30 ± 3	118 ± 3	6.38×10^{1} (±4.81)	2.56×10^{3} ($\pm9.55\times10^{1}$)
		0.12	31 ± 7	309 ± 4		
		0.25	35 ± 6	588 ± 23		
		0.50	32 ± 4	1380 ± 21		
		1.00	66 ± 5	3130 ± 299		
		2.00	156 ± 14	5018 ± 203		

[a] Results are from assays without S9 added. Acetone was used as the dilution solvent and as the solvent control for all compounds except 1,3-dichloropropanone and 1,1,3,3-tetrachloropropanone (TA98), where distilled H_2O and dimethyl sulfoxide were used, respectively.

[b] Initial slope of the dose–response curve (± standard error of the slope).

[c] Determined by gas chromatography with halogen-specific detector.

[d] As indicated by the manufacturer (Fluka Chemical Co.).

[e] Determined by GC/MS.

[f] Determined by proton NMR.

NS, not significant (i.e., no evidence of dose-related increase of 2-fold or greater above background).

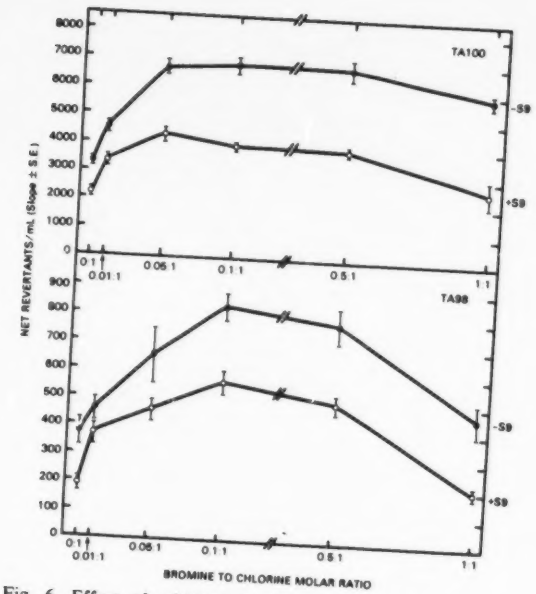

Fig. 6. Effect of addition of different levels of potassium bromide during humic acid chlorination on levels of mutagenicity formed.

highest mutagenic responses with chlorinated humic acid were previously obtained using these assay conditions (Meier et al., 1983). The assay results (Table 2) confirm the direct-acting mutagenicity for strain TA100 for all of the compounds tested. Except for the two dichloropropanone isomers, these compounds were also found to be direct-acting mutagens for strain TA98. The mutagenic potencies, which were calculated from the initial slopes of the dose–response curves, were used to estimate the expected contribution of these compounds to the total mutagenicity present in the chlorinated humic acid solution (Table 3). Assuming simple additivity of response, these data indicate that the identified mutagenic constituents which have been quantified thus far account for approximately 7% of the total TA100 mutagenicity, and about 2% of the TA98 mutagenicity.

One explanation for the failure to account for the mutagenicity present on the basis of the response of the identified mutagens might be that the components when present as a mixture may act synergistically to produce a mutagenic response higher than that expected based on simple additivity. In order to test this possibility, the compounds listed in Table 1 (except 1-chloropro-

TABLE 3

CONTRIBUTION OF IDENTIFIED MUTAGENIC COMPOUNDS TO THE TOTAL MUTAGENICITY OF A CHLORINATED HUMIC ACID SAMPLE

Compound	Theoretical contribution to mutagenicity (net revertants/ml) [a]		Total mutagenicity (%)	
	TA98	TA100	TA98	TA100
Dichloroacetonitrile	0.12	3.42	0.03	0.13
1,1-Dichloropropanone	–	0.04	–	< 0.01
1,3Dichloropropanone	–	17.08	–	0.67
1,1,1-Trichloropropanone	0.86	7.38	0.21	0.29
1,1,3-Trichloropropanone	ND	1.23 [b]	ND	0.05
1.1.3.3-Tetrachloropropanone	1.47	20.31	0.36	0.80
Pentachloropropanone	3.08	26.71	0.75	1.05
3,3Dichloropropenal	0.01	0.35	< 0.01	0.01
2,3,3-Trichloropropenal	2.23	84.60	0.54	3.53
Sum of identified compounds	7.77	166.12	1.88	6.55
Total sample activity [c]	412 ± 25	2537 ± 196	(100.00)	(100.00)

[a] Determined by multiplying the concentration (μg/ml) of the compound in the chlorinated humic acid solution (Table 1) by the specific mutagenicities (net revertants/μg) of the compound (Table 2).

[b] Based on a specific mutagenicity of 2.46×10^1 net revertants/μg, estimated from the data of Douglas et al. (1984).

[c] Mean slope value ± standard deviation of the mean, based on results of 8 independent assays for TA100 and 2 independent assays for TA98 of the same chlorinated humic acid solution used in compound quantifications.

ND, no data.

panone, 1,1,3-trichloropropanone, and 1,1,1,3-tetrachloropropanone which were not available) were added together in distilled water in the amounts at which they were determined to be originally present. In addition, a second mixture was made by adding the compounds to the original solution of chlorinated humic acid. The latter was done to test for potential synergism with components not yet identified. The results of testing these two mixtures for mutagenicity in the Ames test are shown in Fig. 5. The reconstructed sample of identified components in water did produce a dose-related response with strain TA100 which was about 1.4 times higher than the solvent control response at the highest dose tested, but there was no evidence of an increasing response with strain TA98. The slope from the TA100 dose–response curve was 195 net revertants/ml vs. a slope of 2344 net revertants/ml for the original sample, thus accounting for approximately 8% of the TA100 activity in the original sample. The TA100 response as a result of testing the mixture of compounds after addition to the chlorinated humic acid sample was slightly higher than expected based on additivity, giving a slope of 2651. However, the differences in activity were within the standard errors of the slopes. The TA98 response of this mixture was essentially identical to that of the original sample (407 vs. 403 net revertants/ml).

Influence of bromide during chlorination

Since a number of brominated and mixed bromo- and chloro-containing halogenated compounds had been shown to be formed when bromide was present during humic acid chlorination (Coleman et al., 1984), it was of interest to know what influence bromide might have on the level of mutagenicity formed. The results in Fig. 6 show that the mutagenicity for both strains TA98 and TA100 increased with the addition of increasing amount of bromide up to a bromide to chlorine molar ratio of about 0.1 : 1. The level of enhancement reached a maximum of about 2-fold and then declined at higher bromide concentrations.

Discussion and conclusions

In addition to the compounds previously identified and quantified in methylene chloride and ether extracts of chlorinated humic acid solutions (Coleman et al., 1984), a number of other compounds have now been quantified in more highly concentrated ether extracts. These include: monochloroacetonitrile, monochloropropanone, 1,3-dichloropropanone, 1,1,3-trichloropropanone, hexachlorocyclopentadiene, 2,4,6-trichlorophenol, hexachloroethane, 3,3-dichloropropenal, and 2,3,3-trichloropropenal. Many of these compounds have been previously identified in finished drinking water (Table 1). Based on the results of testing the identified compounds either individually (Table 2, Figs. 2–4), or as mixtures in quantities intended to reconstruct the chlorinated humic acid solution (Fig. 5), it is apparent that only about 7–8% of the TA100 mutagenic activity, and less than 2% of the TA98 mutagenic activity, can be accounted for by compounds identified thus far. Failure to account for a greater proportion of the activity cannot be due to losses during solvent extraction and extract concentration since essentially all of the mutagenic activity was recovered in the same ether extract used for GC injection (Fig. 1). However, other experiments have shown that high temperaturees during GC injection can result in degradation of the mutagenic activity (Kopfler et al., 1984). Furthermore, problems with loss of activity during "hot" GC injection are not solved by direct on-column application of the extract without heat, since essentially no mutagenic activity could be recovered by post-GC column trapping using either method of sample introduction (Kopfler et al., 1984). The poor recovery may be due to the fact that the mutagenic activity of chlorinated humic acid sample is primarily nonvolatile in nature (Meier et al., 1983), and thus would not seem to be readily amenable to entering the gaseous phase during GC separations. A number of other compounds, many of unknown mutagenicity, have been detected but not quantified, in a steam distillate fraction of chlorinated humic acids (Kopfler et al., 1984), but for the same reasons as stated above, it is unlikely that these other compounds contribute significantly to the overall mutagenicity. Current efforts are aimed at isolation of the mutagenic activity by high-pressure liquid chromatography (HPLC) in hopes of yielding a less complex sample for chemical analysis. The HPLC method should also help to de-

termine whether a major portion of the mutagenicity can be attributed to any single compound. However, it is probable that the eventual identification of the mutagens will require the development of derivatization techniques which will make the mutagens more gas chromatographable.

Of the 19 compounds identified and quantified thus far, 8 are chlorinated propanones. All of these compounds have been previously identified as constituents of pulp and paper mill chlorination stage effluents (Douglas et al., 1983; Kringstad et al., 1981). The formation of chloropropanones following humic acid chlorination was previously postulated based on similarities in humic and lignitic structures (Meier et al., 1983). The mutagenic activities of several of these compounds in the Ames test have been reported by others, as indicated in Table 1. In the present study, 1,1-dichloropropanone and 1,1,1-trichloropropanone were also found to be mutagenic to Salmonella (Figs. 2 and 3). The latter compound in particular has received interest as a contaminant of drinking water because of its potential as a precursor in chloroform formation (Suffet et al., 1976; Gurol et al., 1983).

Recently, Kringstad et al. (1983) have also reported the formation of 1,3-dichloropropanone, 1,1,3,3-tetrachloropropanone, pentachloropropanone, hexachloropropanone, and 2-chloropropenal following chlorination of humic acid. These workers suggested that 2-chloropropenal in particular may be responsible for a significant part of the mutagenic activity in chlorinated humic acid, although no data were presented to support this claim. In the present study, 2-chloropropenal was synthesized in an attempt to identify and quantify this compound. This compound was not detected in the sample extracts (Table 1), and thus could not be quantified. However, we have qualitatively detected 2-chloropropenal in a steam distillate fraction of chlorinated humic acid (Kopfler et al., 1984). Kringstad et al. (1983) reported the concentration of 2-chloropropenal at 32.8, 43.0, and 44.6 ng/mg organic carbon upon chlorination of 3 different humic materials using the same chlorine to carbon ratio as used in our work, but using an organic carbon concentration 13.6-fold lower than that used here. Correcting for the difference in initial organic carbon, the amount of 2-chloropropenal expected to be formed under our chlorination conditions, based on the highest of the 3 levels reported, would be 44.6 μg/l. This level would be just below our detection limit (approximately 50 μg/l). Based on this level of the compound in our sample, and assuming a potency of 1.25×10^3 TA100 revertants/μg (calculated from the data of Rosen et al., 1980), the contribution of 2-chloropropenal to the total mutagenicity present would be minor (about 2.5%), although significant in comparison to the estimated contributions of other compounds identified and quantified to date. Although 2-chloropropenal was not detected, two other chloropropenals, 3,3-dichloropropenal and 2,3,3-trichloropropenal, were identified and quantified. The mutagenic activity of 3,3-dichloropropenal had not been previously reported but was determined to be 5.83 TA100 revertants/μg, or 0.72 TA100 revertants/nmole. This potency value is 144-fold lower than that reported for 2,3-dichloropropenal by Rosen et al. (1980). The mutagenicity of 2,3,3-trichloropropenal was found to be 407 TA100 revertants/nmole. This agrees reasonably well with the value of 224 TA100 revertants/nmole reported by Rosen et al. (1980). It is notable that the latter compound, which was present at the lowest level of any compound thus far quantified, contributed more than half of the accountable TA100 mutagenic activity. This finding points out one of the major difficulties in trying to assess the relative importance of any particular compound in terms of the total mutagenicity of a complex mixture. Because of the wide variation in mutagenic potency which is known to exist among chemicals, and which is also evident for the compounds identified in the present study (Table 2), a component which seems insignificant in terms of its contribution to the mass of the mixture should not be overlooked in terms of the level of mutagenicity it represents.

Some interesting structure–activity relationships are apparent from the data in Table 3 regarding the mutagenic potency of chloropropanones and chloropropenals. When chlorine substituents are present at both the 1 and 3 carbon positions of propanone, the mutagenic potency decreases with increasing chlorine substitutions. This is in agreement with the findings of Douglas et al. (1983). However, the reverse pattern is ap-

parent when only one of the available carbons is chlorinated, since 1,1,1-trichloropropanone is about 25 times more potent for TA100 than 1,1-dichloropropanone (Figs. 2 and 3), and monochloropropanone is non-mutagenic (Cheh and Carlson, 1981).The potency observed with chlorine substituents at both carbon positions is substantially higher than for the corresponding molecule with only one carbon chlorinated. For example, 1,3-dichloropropanone was found to be 3000 times more mutagenic for TA100 than 1,1-dichloropropanone (Table 2). This finding may be related to the fact that chlorine substitution at both the 1 and 3 carbon positions renders the molecule bifunctional, allowing for alkylation from both carbons with nucleophilic sites on DNA via substitution reactions. Presumably, this would result in intra- and interstrand DNA cross-links.

With regard to the activity of chloropropenals, it is obvious from the present data and the results previously reported by Rosen et al. (1980) and Lutz et al. (1982) that the presence of chlorine at the α-carbon position greatly enhances the mutagenicity of the compound. 2-Chloropropenal, and presumably other halopropenals with substituents in the α-carbon position, have been postulated by Rosen et al. (1980) to react with DNA by cross-linking at nucleophilic sites via Schiff-base formation at the aldehyde carbon and Michael addition at the activated double bond between the α and β carbons. This bifunctional characteristic is apparently greatly reduced in the case of 3,3-dichloropropenal presumably because of reduced electrophilicity at the β carbon in comparison to the situation with 2,3-dichloropropenal, where the electron-withdrawing effect of the α-carbon chlorine substituent enhances the electrophilicity at the β-carbon position. Thus, both for chlorinated propanones and chlorinated propenals, bifunctionality appears to be an important factor in the mutagenic potency of the compounds.

In previous work, it was found that when bromide was present during chlorination of humic acid, as is the case in most water sources used for drinking water, a variety of brominated organic by-products appeared (Coleman et al., 1984). Because many brominated compounds are more mutagenic than the corresponding chlorinated ones, it was of interest to see whether the level of mutagenicity would be increased by the addition of varying amounts of bromide. The results (Fig. 6) indicate that up to a 2-fold increase in activity is seen depending on the level of bromide present. The implication of these results is that the concentration of bromide in water may have a significant impact on the levels of mutagenicity present after chlorination. It would be of interest to investigate the validity of this hypothesis by studying mutagenicity levels in drinking water from areas with widely differing bromide content, but similar organic content in the source waters.

In conclusion, although a number of mutagenic chemicals have been identified in chlorinated humic acid solutions, the large majority of the mutagenic activity remains uncharacterized. The characterization of the mutagenicity appears to be limited by problems in the analytical methodologies. Efforts aimed at isolation and derivatization of the responsible mutagens should help resolve the problems. The examination of humic acid chlorination by-products still appears to be a viable approach to the identification of the compounds responsible for the mutagenicity of chlorinated drinking water. Once such identifications are made, an evaluation of the potential health risks posed by the presence of these compounds in drinking water can be undertaken.

Acknowledgements

We are grateful to Ms. Linda Papa and Dr. John Loper for their helpful comments on the manuscript. We also thank Pat Underwood for typing the manuscript.

The research described in this article has been reviewed by the Health Effects Research Laboratory and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

References

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31, 347–363.

Bull, R.J. (1982) Experimental methods for evaluating the health risks associated with organic chemicals in drinking water, Toxicol. Environ. Chem., 6, 1–17.

Bull, R.J., M. Robinson, J.R. Meier and J. Stober (1982) The use of biological assay systems to assess the relative carcinogenic hazard of disinfection byproducts, Environ. Health Perspect., 46, 215–227.

Cheh, A.M., and R.E. Carlson (1981) Determination of potentially mutagenic and carcinogenic electrophiles in environmental samples, Anal. Chem., 53, 1001–1006.

Cheh, A.M., J. Skochdopole, P. Koski and L. Cole (1980) Nonvolatile mutagens in drinking water; production by chlorination and destruction by sulfite, Science, 207, 90–92.

Christman, R.F., J.D. Johnson, F.K. Pfaender, D.L. Norwood and M.R. Webb (1980) Chemical identification of aquatic humic chlorination products, in: R.L. Jolley et al. (Eds.), Water Chlorination: Environmental Impact and Health Effects, Vol. 3, Ann Arbor Science, MI, pp. 75–84.

Coleman, W.E., J.W. Munch, W.H. Kaylor, R.P. Streicher, H.P. Ringhand and J.R. Meier (1984) GC/MS analysis of mutagenic extracts of aqueous chlorinated humic acid — a comparison of the by-products to drinking water contaminants, Environ. Sci. Technol., 18, 674–681.

deGreef, E., J.C. Morris, C.F. van Kreijl and C.F.H. Morra (1980) Health effects in the chemical oxidation of polluted waters, in: R.L. Jolley et al. (Eds.), Water Chlorination: Environmental Impact and Health Effects, Vol. 3, Ann Arbor Science, MI, pp. 913–924.

Douglas, G.R., E.R. Nestmann, A.B. McKague, O.P. Kamra, E.G.-H. Lee, J.A. Ellenton, R. Bell, D. Kowbel, V. Liu and J. Pooley (1983) Mutagenicity of pulp and paper mill effluent: a comprehensive study of complex mixtures, in: M. Waters, S. Sandhu, L. Claxton, J. Lewtas, S. Nesnow and N. Chernoff (Eds.), Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III, Plenum, New York, pp. 431–459.

Douglas, G.R., E.R. Nestmann, A.B. McKague, R.H.C. San, E.G.-H. Lee, V.W. Liu-Lee and D.J. Kowbel (1984) Determination of potential hazard from pulp and paper mills: Mutagenicity and chemical analysis, in: H.F. Stich (Ed.), Carcinogens and Mutagens in the Environment, Vol. IV, The Workplace, CRC Press, in press.

Fallon, R.D., and C.B. Fliermans (1980) Formation of nonvolatile mutagens by water chlorination; persistence and relationship to molecular weight of organic material in water, Chemosphere, 9, 385–391.

Gurol, M.D., A. Wowk, S. Myers and I.W. Suffet (1983) Kinetics and mechanism of haloform formation from trichloroacetone, in: R.L. Jolley et al. (Eds.), Water Chlorination: Environmental Impact and Health Effects, Vol. 4, Ann Arbor Science, MI, pp. 269–284.

Haworth, S., T. Lawlor, K. Mortelmans, W. Speck and E. Zeiger (1983) Salmonella mutagenicity test results for 250 chemicals, Environ. Mutagen., 5, 3–142.

Kool, H.J., C.F. van Kreijl and B.C.J. Zoeteman (1983) Toxicology assessment of organic compounds in drinking water, in: C.P. Straub (Ed.), CRC Critical Reviews in Environmental Control, Vol. 12, CRC Press, pp. 307–358.

Kopfler, F.C., H.P. Ringhand, W.E. Coleman, and J.R. Meier (1984) Reactions of chlorine in drinking water, with humic acids and in vivo, in: R.L. Jolley (Ed.), Water Chlorination: Environmental Impact and Health Effects, Vol. 5, in press.

Kringstad, K.P., P.O. Ljungquist, F. deSousa and L.M. Strömberg (1981) Identification and mutagenic properties of some chlorinated aliphatic compounds in the spent liquor from kraft pulp chlorination, Environ. Sci. Technol., 15, 562–566.

Kringstad, K.P., P.O. Ljungquist, F. deSousa and L.M. Strömberg (1983) On the formation of mutagens in the chlorination of humic acid, Environ. Sci. Technol., 17, 553–555.

Loper, J.C. (1980) Mutagenic effects of organic compounds in drinking water, Mutation Res., 76, 241–268.

Loper, J.C., M.W. Tabor, L. Rosenblum and J. DeMarco (1985) Continuous removal of both mutagens and mutagen-forming potential by an experimental full-scale granular activated carbon treatment system, Environ. Sci. Technol., 19, 333–339.

Lutz, D., E. Eder, T. Neudecker and D. Henschler (1982) Structure–mutagenicity relationship in α,β-unsaturated carbonylic compounds and their corresponding allylic alcohols, Mutation Res., 93, 305–315.

Maruoka, S., and S. Yamanaka (1980) Production of mutagenic substances by chlorination of waters, Mutation Res., 79, 381–386.

Meier, J.R., R.D. Lingg and R.J. Bull (1983) Formation of mutagens following chlorination of humic acid: a model for mutagen formation during drinking water treatment, Mutation Res., 118, 25–41.

Nestmann, E.R. (1983) Mutagenic activity of drinking water, in: H.F. Stich (Ed.), Carcinogens and Mutagens in the Environment, Vol. 3, CRC Press, FL, pp. 137–147.

Nestmann, E.R., I. Chu, D.J. Kowbel and T.I. Matula (1980) Short-lived mutagen in Salmonella produced by reaction of trichloroacetic acid and dimethyl sulfoxide, Can. J. Genet. Cytol., 22, 35–40.

Rook, J.J. (1976) Haloforms in drinking water, J. AWWA, 68, 168–172.

Rosen, J.D., Y. Segall and J.E. Casida (1980) Mutagenic potency of haloacroleins and related compounds, Mutation Res., 78, 113–119.

Simmon, V.F., I. Kauhanen and R.G. Tardiff (1977) Mutagenic activity of chemicals identified in drinking water, in; D. Scott, B.A. Bridges and F.H. Sobels (Eds.), Progress in Genetic Toxicology, Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 249–258.

Stevens, A.A., C.J. Slocum, D.R. Seeger and G.C. Robeck (1978) Chlorination of organics in drinking water, in: R.L. Jolley (Ed.), Water Chlorination: Environmental Impact and Health Effects, Vol. 1, Ann Arbor Science, MI, pp. 77–104.

Suffet, I.H., L. Brenner and B. Silver (1976) Identification of 1,1,1-trichloroacetone (1,1,1-trichloropropanone) in two drinking waters: a known precursor in haloform reaction, Environ. Sci. Technol., 10, 1273–1275.

Tabor, M.W. (1983) Structure elucidation of 3-(2-chloroethoxy)-1,2-dichloropropene, a new promutagen from an old drinking water residue, Environ. Sci. Technol., 17, 324–329.

Waskell, L. (1978) A study of the mutagenicity of anesthetics and their metabolities, Mutation Res., 57, 141–153.

Wyandotte Chemicals Corporation (1965) Preparation of 1,1,1-trihalo-2-ketones, Neth. Appl. 6408831, Chem. Abstr., 63, 2900d.